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(54) Title: MUCOSAL IMMUNIZATION USING PARTICLE-MEDIATED DELIVERY TECHNIQUES

(57) Abstract

A method for eliciting an immune response against a virus or other pathogens in a mammalian subject is provided. The method includes the steps of providing a particle coated with DNA encoding an antigen derived from a virus, and then administering the particle to mucosal tissue of the mammal using particle-mediated delivery techniques, whereby the particle is delivered into a recipient cell in said tissue. The technique is capable of inducing an effective mucosal immune response in mammals.

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# MUCOSAL IMMUNIZATION USING PARTICLE-MEDIATED DELIVERY TECHNIQUES

## CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

## STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

## BACKGROUND OF THE INVENTION

### Technical Field

The present invention relates generally to methods of immunization. More particularly, the invention pertains to the delivery of nucleic acid molecules or peptide antigens into mucosal tissue using particle-mediated delivery techniques.

Conventional vaccination strategies generally involve administration of either "live" or "dead" vaccines. Ertl et al. (1996) *J. Immunol.* 156:3579-3582. The so-called live vaccines include attenuated microbes and recombinant molecules based on a living vector. The dead vaccines include those based on killed whole pathogens, and subunit vaccines, e.g., soluble pathogen subunits or protein subunits. Live vaccines are generally successful in providing an effective immune response in immunized subjects; however, such vaccines can be dangerous in immunocompromised or pregnant subjects, can revert to pathogenic organisms, or can be contaminated with other pathogens. Hassett et al. (1996) *Trends in Microbiol.* 8:307-312. Dead vaccines avoid the safety problems associated with live vaccines; however such vaccines often fail to provide an appropriate and/or effective immune response in immunized subjects.

More recently, direct injection of DNA and mRNA into mammalian tissue for the purpose of eliciting an immune response has been described. See, e.g., U.S. Patent No. 5,589,466. The method, termed "naked DNA immunization," has been reported to elicit both humoral and cell-mediated immune responses following DNA delivery to muscle. For example, sera from mice immunized with a human immunodeficiency virus type 1 (HIV-1) DNA construct encoding the envelope glycoprotein, gp160, were reported to react with recombinant gp160 in immunoassays and lymphocytes from the injected mice were shown to proliferate in response to recombinant gp120 (Wang et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:4156-4160), and mice immunized with a plasmid containing a genomic copy of the human growth hormone (hGH) gene demonstrated a humoral immune response (Tang et al. (1992) *Nature* 356:152-154).

Likewise, intramuscular injection of DNA encoding influenza nucleoprotein has been shown to elicit a CD8+ CTL response that can protect mice against subsequent lethal challenge with virus. Ulmer et al. (1993) *Science* 259:1745-1749. Immunohistochemical studies of the injection site revealed that the DNA was taken up by myeloblasts, and cytoplasmic production of viral protein could be demonstrated for at least six months. Therefore, these immunization techniques can be used to provide for the *in vivo* synthesis of antigenic proteins in a manner that is consistent with natural infection. Such endogenous production allows for processing of the antigens along the classical MHC class I pathway and presentation to CD8+ T lymphocytes, as well as uptake and presentation of soluble proteins by MHC class II molecules to CD4+ T lymphocytes. These features induce both cellular and humoral immune responses, allowing nucleic acid immunization to provide the immunogenic advantages of live vaccines without the concomitant safety concerns. However, the technique of injection of naked DNA into muscle is relatively inefficient and requires much more DNA than other DNA

vaccination approaches.

A number of delivery techniques can be used to deliver nucleic acids for immunizations, including particle-mediated (gene gun) techniques which accelerate nucleic acid-coated microparticles directly into the interior of cells in the target tissue. Gene gun-based nucleic acid immunization has been shown to elicit both humoral and cytotoxic T lymphocyte immune responses following epidermal delivery of nanogram quantities of DNA. Pertmer et al. (1995) *Vaccine* 13:1427-1430. Particle-mediated delivery techniques have been compared to other types of nucleic acid inoculation, and found markedly superior. Fynan et al. (1995) *Int. J. Immunopharmacology* 17:79-83, Fynan et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:11478-11482, and Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519-9523. Such studies have investigated particle-mediated delivery of nucleic acid-based vaccines to both superficial skin and muscle tissue. One possible reason for the markedly better results achieved with the gene gun is that the DNA is delivered intracellularly as opposed to the extracellular delivery by intramuscular injection.

The immunity mechanisms provided by humoral and mucosal immunity systems differ significantly. Mucosal immunity provides an important first line of defense in protection against pathogens which enter through mucosal tissues. The mucosal surfaces of the gastrointestinal, respiratory and genitourinary tracts are continuously exposed to foreign antigen, including potentially infectious bacterial, viral and sometimes parasitic organisms. Mucosal immune responses may protect against such challenges, and have distinct and specialized characteristics. Holmgren et al. (1994) *Am. J. Trop. Med. Hyg.* 50:42-54. Mucosal immunity includes both a humoral (antibody) response and a cytotoxic T lymphocyte (CTL) response, similar to non-mucosal immunity except localized to mucosal tissue.

The current dogma holds as follows. 1. The principal

immunoglobin produced by the mucosal immune system is secretory IgA, which is the most abundant immunoglobin class in humans. 2. Specialized antigen uptake cells in the Peyer's Patches of intestinal tract or nasopharyngeal lymphoid tissues, termed microfold or M cells, transport antigen to the underlying mucosal associated lymphoid tissues (MALT). 3. In other areas of the mucosal epithelium, such as the pseudo-stratified airway epithelium, dendritic cells serve as antigen-presenting cells and migrate to local lymph nodes or MALT. Antigen processing and presentation occurs in the MALT, resulting in activation of antigen-specific IgA B cells. The subsequent trafficking and recirculation of the activated IgA-B cells to other components of the mucosal immune system, e.g., the respiratory, intestinal and genital tracts, provides for disseminated local mucosal IgA responses throughout the "Common Mucosal System." Thus, the mucosal immune system is uniquely suited to respond to the types of antigenic challenge encountered by mucosal surfaces, and may provide the most effective type of immune response against pathogens that initially infect or enter the body through mucosal surfaces. It is difficult to achieve effective mucosal immune response using most prior art techniques.

## BRIEF SUMMARY OF THE INVENTION

The present invention provides an effective method for eliciting an immune response in a mammalian subject using mucosal immunization and particle-mediated delivery techniques.

Accordingly, in one embodiment, the invention is drawn to a method for eliciting a mucosal immune response **or a systemic immune response** against a virus in a mammalian subject. The method includes the steps of (a) providing a particle coated with a nucleotide sequence encoding an antigen derived from the virus, wherein the nucleotide sequence is operably linked to control sequences that

direct the expression thereof in a suitable recipient cell; and (b) administering the particle to mucosal tissue of the mammal using particle-mediated delivery techniques, whereby the particle is delivered into a recipient cell in said tissue, and the nucleotide sequence expressed at sufficient levels to elicit a mucosal immune response against said antigen.

In another embodiment, a method includes the steps of (a) providing a particle coated with an antigen derived from a virus; and (b) administering the particle to mucosal tissue of the mammal using particle-mediated delivery techniques, whereby the particle is delivered into a recipient cell in said tissue.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

## BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 is a schematic representation of the hemagglutinin (HA) expression vector pWRG1638. This plasmid vector was constructed from pWRG7054, a mammalian expression vector based on a pUC19 backbone, and thus contains the cytomegalovirus (CMV) immediate early transcriptional enhancer, promoter and intron A regulatory elements, and the polyA signal of bovine growth hormone, operably linked to the full length cDNA encoding the HA gene from swine influenza virus A/Swine/Indiana/1726/88 (H1N1).

Figure 2 depicts the geometric mean titers of nasal viral shedding profiles in porcine subjects after challenge with the swine influenza virus A/Swine/Indiana/1726/88 (Sw/IN) as described in Example 1. The animals were vaccinated using nucleic acid immunization by a prime and booster administration with: a control plasmid DNA (open squares); the pWRG1638 construct to the epidermis (open diamond); the pWRG1638 construct to mucosal tissue (open triangles); or the pFluNP construct to epidermis (open

circles). Control animals were vaccinated using parenteral injection by a prime and booster administration with a commercial inactivated whole virus vaccine (crossed squares). All animals were challenged two weeks after the booster immunization.

## DETAILED DESCRIPTION OF THE INVENTION

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular antigens or to antigen-coding nucleotide sequences. It is also understood that different applications of the disclosed methods may be tailored to the specific needs in the art. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an antigen" includes a mixture of two or more such agents, reference to "a particle" includes reference to mixtures of two or more particles, reference to "a recipient cell" includes two or more such cells, and the like.

### A. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. The following terms are intended to be defined as indicated below.

An "antigen" refers to any agent, generally a macromolecule, which can elicit an immunological response in an individual. The immunological response may be

mediated by B- and/or T-lymphocytic cells. The term may be used to refer to an individual macromolecule or to a homogeneous or heterogeneous population of antigenic macromolecules. As used herein, "antigen" is generally used to refer to a protein molecule or portion thereof which contains one or more epitopes.

A "B cell epitope" generally refers to the site on an antigen to which a specific antibody molecule binds. The identification of epitopes which are able to elicit an antibody response is readily accomplished using techniques well known in the art. See, e.g., Geysen et al. *Proc. Natl. Acad. Sci. USA* (1984) 81:3998-4002 (general method of rapidly synthesizing peptides to determine the location of immunogenic epitopes in a given antigen); U.S. Patent No. 4,708,871 (procedures for identifying and chemically synthesizing epitopes of antigens); and Geysen et al., *Molecular Immunology* (1986) 23:709-715 (technique for identifying peptides with high affinity for a given antibody).

"T cell epitopes" are generally those features of a peptide structure capable of inducing a T cell response. In this regard, it is accepted in the art that T cell epitopes comprise linear peptide determinants that assume extended conformations within the peptide-binding cleft of MHC molecules, (Unanue et al. (1987) *Science* 236:551-557). As used herein, a T cell epitope is generally a peptide having about 3-5, preferably 5-10 or more amino acid residues.

"Gene delivery" refers to methods or systems for reliably delivering foreign DNA into host cells. Such methods can result in the expression of the foreign DNA in the host cells.

A "nucleotide sequence" or a "nucleic acid molecule" refers to single or double stranded DNA and RNA sequences. The term captures molecules that include any of the known base analogues of DNA and RNA.

A "coding sequence" or a sequence which "encodes" a

particular polypeptide antigen, is a nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences.

The term DNA "control sequences" refers collectively to promoter sequences, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a recipient cell. Not all of these control sequences need always be present so long as the selected gene is capable of being transcribed and translated in an appropriate recipient cell. The control sequences for eukaryotes and prokaryotes can differ significantly, and for the present invention eukaryotic, and preferably, mammalian or mammalian virus control sequences are most suitable.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

B. General Methods

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process parameters as such may, of course, vary. It is also to be understood that the

terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

Although a number of methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

In accordance with the present invention, a method is defined for achieving mucosal immunity **and/or systemic immunity** against an antigen for a pathogen which normally enters the subject body through mucosal tissue. The mucosal immunity, which is contrasted to the humoral immunity obtained through prior DNA vaccination protocols, is achieved by the intracellular delivery of the DNA directly to target mucosal tissues. It has been found that delivery of DNA encoding a pathogenic antigen into the mucosal tissue of a mammal will result in a mucosal immunity expressed by mucosal tissues of the patient including those quite distant from the tissues into which the DNA vaccine is delivered. Prior work has revealed some irregularity in the production of distal mucosal immune response, but the method described here has been found to produce a response in distal mucosal tissues. This enables the DNA vaccine to be delivered to the patient at the site most convenient for delivery of the DNA vaccine, whether or not that site where mucosal tissue is the preferred route of entry of the particular pathogen against which the patient is to be immunized. **A systemic immune response as used herein may include a cell-mediated immune response characterized by peripheral blood CTL or a humoral immunity characterized by increased levels of circulating antibodies, such as IgG, in post immunization blood sera.**

In particular, it has been found that the tongue and the buccal tissue of the interior of the mouth, or cheek, are the most convenient tissues into which to direct a particle-mediated DNA vaccine delivery protocol. The tissues are readily accessible through relatively non-

invasive procedures. It has also been found that both tongue and buccal tissue are capable of engendering a sufficient immune response to introduce mucosal immunity by antigen encoding DNA delivered to these tissues. It has been found that the delivery of antigen encoding DNA to the cheek or buccal tissues results in a systemic mucosal immune response shared by **?immune?** mucosal tissues throughout the body.

The present invention provides a method for eliciting, in a mammalian subject, an immune response against mucosally transmitted pathogens using nucleic acid immunization and particle-mediated delivery techniques. The method can thus be used in a variety of mammalian subjects to provide a suitable immune response against infection by a pathogen which would normally enter the subject through a mucosal tissue. Mucosal tissues are the preferred entry site into the body for a wide variety of pathogens. Pathogens which enter the body through mucosal tissues include Human Pappiloma Viruses (HPV), HIV, HSV2/HSV1, influenza virus (types A, B, and C), Polio virus, RSV virus, Rhinoviruses, Rotaviruses, Hepatitis A virus, Norwalk Virus Group, Enteroviruses, Astroviruses, Measles virus, Para Influenza virus, Mumps virus, Varicella-Zoster virus, Cytomegalovirus, Epstein-Barr virus, Adenoviruses, Rubella virus, Human T-cell Lymphoma type I virus (HTLV-I), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis D virus, Pox virus, Marbug and Ebola; bacteria including *M. tuberculosis, Chlamydia, N. Gonorrhea, Shigella, Salmonella, Vibrio Cholera, Treponema pallidua, Pseudomonas, Bordetella pertussis, Brucella, Franciscella tulorensis, Helicobacter pylori, Leptospria interrogaus, Legionella pneomophila, Yersinina pestis*, Streptococcus (types A and B), *Pneumococcus, Meningococcus, Hemophilus influenza* (type b), *Toxoplasma gondic, Complylobacteriosis, Moraxella catarrhalis, Legionella pneumophlia, Pseudomonas aeruginosa, Donovanosis*, and *Actinomycosis*; fungal pathogens including Candidiasis and

Aspergillosis; parasitic pathogens including Taenia, Flukes, Roundworms, Amebiasis, Giardiasis, Cryptosporidium, Schistosoma, Pneumocystis carinii, Trichomoniasis and Trichinosis. The present invention can be used to provide a suitable immune response against numerous veterinary diseases, such as Foot and Mouth diseases, Coronavirus, *Pasteurella multocida*, *Helicobacter*, *Strongylus vulgaris*, *Actinobacillus pleuropneumonia*, Bovine viral virus diarrhea (BVDV), *Klebsiella pneumoniae*, *E. coli*, *Bordetella pertussis*, *Bordetella parapertussis* and *brochiseptica*.

The invention is broadly applicable for providing an immune response against any pathogen which would normally enter through mucosal tissue. In the examples below, there is reference to influenza virus and immunodeficiency virus DNA. Both of these are intended only as examples of viruses which enter the body through mucosal tissues. It is here thought that a suitable mucosal immune response can be created following delivery of DNA encoding antigens from these viruses to mucosal tissues. By suitable immune response, it is meant that the methods of the invention can bring about in an immunized subject an immune response characterized by the stimulation and clonal expansion of B and/or T lymphocytes specific for a virus antigen, wherein the immune response can protect the subject against subsequent infection with homologous or heterologous viral strains, reduce viral burden and/or shedding during an infection, bring about resolution of infection in a shorter amount of time relative to a non-immunized subject, or prevent or reduce clinical manifestation of disease symptoms.

Generally, nucleic acid molecules used in the subject methods contain coding regions with suitable control sequences and, optionally, ancillary therapeutic nucleotide sequences. The nucleic acid molecules are prepared in the form of vectors which include the necessary elements to direct transcription and translation in a recipient cell. The nucleic acids may be the entire genome of the virus

less only sequences necessary for viral pathogenicity.

In order to augment an immune response in an immunized subject, the antigen-encoding nucleic acid molecules can be administered in conjunction with ancillary substances, such as pharmacological agents, adjuvants, cytokines, or in conjunction with delivery of vectors encoding cytokines.

More particularly, ancillary therapeutic nucleic acid sequences coding for peptides known to stimulate, modify, or modulate a host's immune response, can be coadministered with the above-described antigens. Thus, genes encoding one or more of the various cytokines (or functional fragments thereof), such as the interleukins, interferons, and colony stimulating factors, will find use with the instant invention. The gene sequences for a number of these substances are known. In one embodiment of the invention, mucosal nucleic acid immunization is coupled with codelivery of one or more of the following immunological response modifiers: IL-2; IL-4; IL-6; IL-10; IL-12; and IFN-$\gamma$.

Modes of carrying out the invention are described more fully below.

Isolation of Genes and Construction of Vectors

Nucleotide sequences selected for use in the present invention can be derived from known sources, for example, by isolating the same from infected cells or viral particles containing a desired gene or nucleotide sequence using standard techniques. The nucleotide sequences for many, if not most, pathogen antigens have been identified to assist in vaccine and therapy design. It is now possible to construct DNA molecules of significant length once DNA sequence information is available.

Once coding sequences for desired antigens have been prepared or isolated, such sequences can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice.

Ligations to other sequences are performed using standard procedures, known in the art.

Selected nucleotide sequences can be placed under the control of regulatory sequences such as a promoter or ribosome binding site (collectively referred to herein as "control" elements), so that the sequence encoding the desired antigen is transcribed into RNA in the host tissue transformed by a vector containing this expression construct.

The choice of control elements will depend on the host being treated and the type of preparation used. Thus, if the host's endogenous transcription and translation machinery will be used to express the proteins, control elements compatible with the particular host will be utilized. In this regard, several promoters for use in mammalian systems are known in the art and include, but are not limited to, promoters derived from SV40, CMV, HSV, RSV, MMTV, among others.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of antigens encoded by the delivered nucleotide sequences. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a coding sequence to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate control and, optionally, regulatory sequences such that the positioning and orientation of the coding sequence with respect to the control sequences allows the coding sequence to be transcribed under the "control" of the control sequences (i.e., RNA polymerase, which binds to the DNA molecule at the control sequences, transcribes the coding sequence). Modification of the

sequences encoding the particular antigen of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it is attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

Conventional mammalian expression vectors and elements can be enhanced for use as DNA vaccines. For example, it has been found that the addition of signal peptide sequences directing secretion of expressed proteins can enhance CTL immune response. The use of a Kozak ATG sequence can enhance the translational efficiency of a DNA vaccine. The inclusion of a mono/poly ubiquitination sequence in the expression vector can enhance the MHC Class I presentation signal while alternatively the use of an invariant chain sequence can enhance MHC Class II presentation signal. The use of such elements is within the abilities of those of skill in the art.

Administration of Nucleic Acid Preparations

Particle-mediated methods for delivering nucleic acid preparations are known in the art. Thus, once prepared and suitably purified, the above-described nucleic acid molecules can be coated onto carrier particles using a variety of techniques known in the art. Carrier particles are selected from materials which have a suitable density in the range of particle sizes typically used for intracellular delivery from a gene gun device. The optimum carrier particle size will, of course, depend on the diameter of the target cells.

For the purposes of the invention, tungsten, gold, platinum and iridium carrier particles can be used. Tungsten and gold particles are preferred. Tungsten

particles are readily available in average sizes of 0.5 to 2.0 μm in diameter. Although such particles have optimal density for use in particle acceleration delivery methods, and allow highly efficient coating with DNA, tungsten may potentially be toxic to certain cell types and may degrade DNA over time. Gold particles or microcrystalline gold (e.g., gold powder A1570, available from Engelhard Corp., East Newark, NJ) will also find use with the present methods. Gold particles provide uniformity in size (available from Alpha Chemicals in particle sizes of 1-3 μm, or available from Degussa, South Plainfield, NJ in a range of particle sizes including 0.95 μm) and reduced toxicity. Microcrystalline gold provides a diverse particle size distribution, typically in the range of 0.5-5 μm.

A number of methods are known and have been described for coating or precipitating DNA or RNA onto gold or tungsten particles. Most such methods generally combine a predetermined amount of gold or tungsten with plasmid DNA, $CaCl_2$ and spermidine. The resulting solution is vortexed continually during the coating procedure to ensure uniformity of the reaction mixture. After precipitation of the nucleic acid, the coated particles can be transferred to suitable membranes and allowed to dry prior to use, coated onto surfaces of a sample module or cassette, or loaded into a delivery cassette for use in particular gene gun instruments.

<u>Administration of Coated Particles</u>

Following their formation, carrier particles coated with either nucleic acid preparations, or peptide or protein antigen preparations, are delivered to mucosal tissue using particle-mediated delivery techniques.

Various particle acceleration devices suitable for particle-mediated delivery are known in the art, and are all suited for use in the practice of the invention. Current particle acceleration device designs employ an

explosive, electric or gaseous discharge to propel coated carrier particles toward target cells. The coated carrier particles can themselves be releasably attached to a movable carrier sheet, or removably attached to a surface along which a gas stream passes, lifting the particles from the surface and accelerating them toward the target. An example of a gaseous discharge device is described in U.S. Patent No. 5,204,253. An explosive-type device is described in U.S. Patent No. 4,945,050. One example of an electric discharge-type particle acceleration apparatus is the ACCELL® instrument (Geniva, Madison, WI), which instrument is described in U.S. Patent No. 5,120,657. Another electric discharge apparatus suitable for use herein is described in U.S. Patent No. 5,149,655. The disclosure of all of these patents is incorporated herein by reference in their entireties.

The coated particles are administered to the subject to be treated in a manner compatible with the dosage formulation, and in an amount that will be effective to bring about a desired immune response. The amount of the composition to be delivered which, in the case of nucleic acid molecules is generally in the range of from 0.001 to 10.0 $\mu$g, more preferably 0.25 to 5.0 $\mu$g of nucleic acid molecule per dose, depends on the subject to be treated. By dose, it is meant to refer to a single event of DNA delivery by gene gun. Using current gene guns, it is common for a single immunization procedure, whether a prime immunization or a boost, to include more than one gene gun dose. For example, a prime might consist of two to six gene gun doses to the tongue. Adding more DNA to each dose, beyond 0.25 to 5$\mu$g, generally does not increase immune response. The additional doses are appropriate to, in essence, treat more tissue. A gene gun design which is capable of treating more tissue in a single operation would lower the number of doses in a single vaccination. In general, however, the total amount of DNA delivered in the entire immunization will be in the range of about 1-30$\mu$g

total for all doses. Often a prime immunization and either one or two boost immunizations will be appropriate to achieve the desired level of immune response. The exact amount necessary will vary depending on the age and general condition of the individual being immunized and the particular nucleotide sequence or peptide antigens selected, as well as other factors. An appropriate effective amount can be readily determined by one of skill in the art upon reading the instant specification.

In the examples described below, over-dosages of DNA have been used. This was done because optimization of dosages for the particular antigens and the particular animals have not yet been done. It has been previously found that mild over-dosing of delivered DNA is not harmful to the immune response and thus, to err in dosing to achieve the desired immune response, it was decided to err on the high side. For a practical nucleotide vaccine for a given antigen, optimization studies would be performed to determine the minimum dosing required and such studies are well within the skill of those in the art.

Thus, an effective amount of the antigens herein described, or rather nucleic acids coding therefor, will be sufficient to bring about a suitable immune response in an immunized subject, and will fall in a single to double digit microgram range of DNA that can be optimized through routine trials for a particular DNA and mammal.

The coated particles are delivered to suitable recipient cells in mucosal tissue in order to bring about mucosal, humoral and/or cellular immune responses in the treated subject.

C. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

## Example 1
## Particle-Mediated Nucleic Acid Immunization Directed to Porcine Mucosal Tissue

In order to assess the effectiveness of particle-mediated nucleic acid immunization of mucosal tissue, the following studies were carried out.

### Experimental subjects

Weanling pigs (10-15 kg) sero-negative for swine influenza by hemagglutination inhibition (HI), (Palmer et al. (1975) U.S. Department of Health, Education and Welfare Immunology Series) and ELISA (Sheerar (1989) *J. Gen. Virol.* 70:3297-3303) were housed in a Biosafety level 2-N facility for immunizations, and then housed in a Biosafety level 3-N facility for viral challenge. The animal subjects were cared for in accordance with the guidelines prescribed by the University of Wisconsin Research Animal Resource Center.

### Viral preparations

An A/swine influenza isolate, A/Swine/Indiana/1726/88 (H1N1) (Sw/IN), was obtained from the influenza repository at the University of Wisconsin School of Veterinary Medicine. The virus was cultured in 10-day-old embryonated hens' eggs and stored at -70 °C as previously described (Sheerar et al., *supra*) except that the allantoic fluid was concentrated by the addition of PEG-8000 to 8%. Precipitated virus was centrifuged at 8000 X g prior to purification on 30-60% sucrose gradients at 24,000 rpm in an SW28 rotor (Beckman).

Plasmid constructs and DNA preparations

The hemagglutinin expression plasmid pWRG1638 depicted in Figure 1 was constructed by ligating the cloned cDNA encoding the HA of swine influenza virus (SW/IN/1726/88) into the mammalian expression cassette pWRG7054. The cDNA synthesis of the HA gene was done in a one-step PCR method according to Wentworth et al. (1994) *J. Virol.* 68:2051-2058. PWRG1638 is a pUC19-based vector and includes the human cytomegalovirus immediate early transcriptional enhancer/promoter (CMVie) to drive transcription of the HA coding region. The plasmid also contains the polyadenylation region from the bovine growth hormone bGH gene (Chapman et al. (1991) *Nucleic Acids Res.* 19:3979-3986). An influenza nucleoprotein (NP) expression plasmid, pFluNP, that encodes the nucleoprotein of influenza A strain PR/8/34 was obtained from Dr. K. Irvine at the National Cancer Institute. All plasmids were propagated in *E.coli* strain XL1-Blue MR. Supercoiled plasmid DNA was prepared on Qiagen columns according to the manufacturer's instructions.

Preparation of coated microparticles

Plasmid DNA was coated onto gold particles normally in the range of 1-3 μm in size (Degussa Corp., South Plainfield, NJ) using techniques described by Eisenbraun et al. (1993) *DNA Cell Biol.* 12:791-797. The DNA-coated gold particles were then loaded into Tefzel® tubing as described in U.S. Patent No. 5,584,807 to McCabe, and the tubing was cut into 1.27 cm lengths to serve as cartridges in the ACCELL® gene gun delivery device. The helium-pulse ACCELL® gene gun device was obtained from Geniva, Madison, WI. In the vaccinations, each 1.27 cm cartridge contained 0.5 mg gold particles coated with 1.25 μg of plasmid DNA.

*In vitro* expression of HA in CHO cells

Chinese hamster ovary (CHO) cells were transfected with the pWRG1638 construct, or with control plasmid

pWRG1630 which codes for the mature form of epidermal growth factor (Andree et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:12188-12192), using the electric discharge ACCELL® gene gun delivery device (Geniva, Madison, WI). In the study, the CHO cells were cultured as monolayers on 22x22 mm glass cover slips. For transfection, growth medium was aspirated and the cells treated as previously described (Christou et al. (1990) *Trends Biotech.* 8:145-151. After transfection, fresh medium was added and the cells were incubated at 37°C overnight. Following incubation, the cells were fixed with a methanol/acetone (50:50 v/v) fixing solution at -20°C, and then air dried. The fixed cells were incubated with a panel of the following monoclonal antibodies which are specific for the HA protein of swine influenza A (SW/IN/1726/88): 3F2c, 1-6b2, 2-15f1 and 7B1b (Sheerar et al., *supra*). Incubation was conducted at room temperature for 60 minutes, after which the fixed cells were washed and incubated with biotinylated goat anti-murine antibodies (Oncogene Sciences, Inc.). The cells were then washed again, and incubated with fluorescein-conjugated streptavidin (Oncogene Sciences, Inc.). Fluorescently labeled cells were visualized using a suitable fluorescence microscope (e.g., a Zeiss Photomicroscope III™ equipped for fluorescence microscopy).

As a result of the study, CHO cells that were transfected with the pWRG1638 construct showed intense staining, indicating that the cells were expressing influenza HA. CHO cells transfected with the pWRG1630 control plasmid were not immunoreactive in the assay.

*In vivo* vaccination studies

Based on the positive results seen in the above-described *in vitro* transfection study, a vaccination trial was initiated using *in vivo* particle-mediated delivery methods. Animal subjects receiving nucleic acid immunizations in the present study included: (1) a first experimental group of three pigs that were vaccinated by

particle-mediated delivery to the epidermis with the NP expression vector pFluNP; (2) a second experimental group of four pigs that were vaccinated by particle-mediated delivery to the epidermis with the HA expression vector pWRG1638; (3) a third experimental group of five pigs that were vaccinated by particle-mediated delivery to the inferior surface of the tongue (mucosal immunization) with the HA expression vector pWRG1638; and (4) a fourth experimental group of four pigs that were vaccinated by particle mediated delivery to the epidermis with a negative control plasmid pWRG3510 (a plant expression vector encoding β-glucuronidase from *E. coli* and which is inactive in mammalian cells). Animals in the first and second experimental groups were immunized using *ACCELL*® gene gun transfer of either the pFluNP, the pWRG1638 construct, or the control plasmid pWRG3510, into the epidermis in different anatomical regions including the dorsal surface of the ear, the inguinal region, and the lateral thoracic region. Treatment typically included six target sites at each location. Hair was removed with clippers prior to treatment of the lateral thoracic region, but other regions were treated without prior preparation. Delivery was conducted at 500 or 600 psi helium pressure. Animals in the third experimental group were immunized using *ACCELL*® gene gun transfer of the pWRG1638 construct into the mucosal tissue of the inferior surface of the tongue using 500 or 600 psi driving gas. In other vaccinations, a fifth experimental group of four pigs received a 2 ml parenteral (intramuscular) injection of a commercial swine influenza vaccine (MaxiVac™-FLU, SyntroVet, Kenexa, KS) as directed by the manufacturer. The MaxiVac™-FLU vaccine is an oil-in-water vaccine containing inactivated whole Influenza A (H1N1) virus. Vaccination consisted of a priming administration followed by a booster injection four weeks later. A sixth experimental group of four pigs was infected with swine influenza and allowed to recover from infection to provide a comparison between protection

afforded by conventional vaccine and by natural infection.

In experimental groups 1-5, serum samples were collected prior to vaccination, prior to booster administration, and one week after booster administration. All blood samples were collected from the superior vena cava. After these serum collections were completed, the animals were challenged with virus, the course of infection monitored, and sera was again collected two weeks after the challenge.

Viral challenge consisted of intranasal instillation of 2 x $10^4$ or 2 x $10^6$ $EID_{50}$ (50% egg infectious dose) of SW/IN virus. Challenged animals were monitored daily for clinical signs of influenza infection (e.g., lethargy, coryza and elevated body temperature). Nasal swabs were collected from each pig on days 1, 3, 5, and 7 post infection, and viral titers were determined by limiting-dilution egg inoculation assays (Wentworth et al. (1994) *J. Virol.* 68:2051-2058). Ten days after challenge, convalescent sera were taken.

Sera from the various experimental groups were analyzed by ELISA and HI assays. ELISA serology was conducted using 200 hemagglutinin (HA) units/well of Sarksyl-disrupted purified SW/IN virus diluted in PBS as described (Sheerar et al., *supra*), with the swine antibodies being measured directly using a goat anti-swine IgG alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, MD). HI assays were performed using previously described techniques (Palmer et al. (1975), *supra*).

The results of the ELISA and HI assays for all six experimental groups are depicted below in Table 1. As can be seen, antibody or HI titers were not detected in any of the experimental groups receiving nucleic acid immunizations four weeks post prime. ELISA titers, ranging from 1:200 to 1:1600, were seen in animals receiving epidermal vaccinations with the NP (pFluNP) and HA (pWRG1638) expression vectors (experimental groups 1 and 2,

respectively) two weeks after the boost, and HI titers ranging from 1:10 to 1:160 were seen in the animals of group 2 vaccinated with the HA construct (pWRG1638). The NP-vaccinated animals (group 1) did not have HI titers, despite high ELISA titers, because the HI assay only detects HA-specific antibodies.

The animals that received mucosal vaccinations with the pWRG1638 HA DNA construct (group 3) had higher ELISA titers (ranging from 1:800 to 1:6400), and lower HI titers (ranging from 1:20 to 1:80), relative to the animals of groups 1 and 2 that received epidermal vaccinations. The animals of group 5 vaccinated with the inactivated whole virus exhibited the highest ELISA and HI titers relative to all other experimental groups, while the group receiving natural infection (group 6) had ELISA and HI titers similar to the groups vaccinated with the pWRG1638 HA DNA construct. Control animals vaccinated with the plant expression vector (group 4) showed no evidence of an anti-influenza immune response.

Table 1

| Type of Reciprocal Vaccination | Animal Number | 4 Week Post-Prime Reciprocal ELISA Titer | 4 Week Post-Prime Reciprocal HI Titer | 2 Week Post-Boost Reciprocal ELISA Titer | 2 Week Post-Boost Reciprocal HI Titer | Post-Challenge HI Titer |
|---|---|---|---|---|---|---|
| Epidermal NP DNA Vaccine | 1 | <100 | <10 | 1600 | <10 | 20 |
| | 2 | <100 | <10 | 800 | <10 | 80 |
| | 3 | <100 | <10 | 1600 | <10 | 80 |
| Epidermal NP DNA Vaccine | 1 | <100 | <10 | 1600 | 10 | 160 |
| | 2 | <100 | <10 | 800 | 20 | 5120 |
| | 3 | <100 | <10 | 800 | 160 | 5120 |
| | 4 | <100 | <10 | 200 | 40 | 1280 |
| Tongue HA DNA Vaccine | 1 | <100 | <10 | 3200 | 80 | 2560 |
| | 2 | <100 | <10 | 3200 | 40 | 5120 |
| | 3 | <100 | <10 | 1600 | 20 | 5120 |
| | 4 | <100 | <10 | 12800 | 80 | 5120 |
| Inactivated Whole Virus | 1 | 6400 | 160 | 32000 | 5120 | ND |
| | 2 | 1600 | 40 | 4000 | 80 | ND |
| | 3 | 6400 | 80 | 8000 | 160 | ND |
| | 4 | 800 | 40 | 32000 | 80 | ND |
| Natural Infection* | 1 | 3200 | 160 | 1600 | NA | 80 |
| | 2 | 800 | 40 | 1600 | NA | 40 |
| | 3 | 1600 | 160 | 1600 | NA | 160 |
| | 4 | 800 | 20 | 6400 | NA | 40 |
| Negative Control | 1 | <100 | <10 | <10 | <10 | 40 |
| | 2 | <100 | <10 | <10 | <10 | 80 |
| | 3 | <100 | <10 | <10 | <10 | 80 |
| | 4 | <100 | <10 | <10 | <10 | 80 |

*The natural infection cohort was bled three weeks after the first infection and two weeks after the second infection; ND-Not determined; NA-Not applicable

As can also be seen by reference to Table 1, the animals vaccinated with the pFluNP construct (group 1) and the animals treated by "natural infection" had post-challenge HI titers ranging from 1:80-1:160. These titers are similar to the HI titers seen in the negative control animals of group 6 that were vaccinated with the pWRG3510 (β-glucuronidase) construct. In contrast, both groups of animals that were vaccinated with the pWRG1638 HA DNA constructs (groups 2 and 3) had HI titers as high as 1:5120 after viral challenge. Even the animal from group 2 that responded poorly to the pre-challenge vaccination in terms of HI titer showed some evidence of a hyperimmune response following viral challenge.

With respect to the levels of protection afforded by the various methods of immunization (e.g., nucleic acid immunization, parenteral vaccination or viral infection), clinical signs of disease (lethargy, coryza and elevated body temperature) were monitored during infection, but did not provide a reliable measure of disease progression. On the other hand, nasal viral titers provided a quantitative indicator for disease progression.

Referring now to Figure 2, animals vaccinated epidermally with the pFluNP DNA construct (group 1) developed high antibody titers to NP, but showed no evidence of protection from viral infection in terms of nasal virus titer. Animals receiving epidermal vaccination with the pWRG1638 HA DNA construct (group 2) became infected and shed lower levels of virus over the course of infection, and resolved infection approximately two days earlier than the control animals of group 6. Animals receiving mucosal vaccination with the pWRG1638 HA DNA construct (group 3) developed weak HI titers, but were able to reduce viral shedding over the seven days of the study. Further, the mucosally vaccinated animals were able to reduce the initial infection, as evidenced by a decrease in the level of shedding by an order of magnitude on days 1 and 3, relative to the epidermally vaccinated animals of

group 2.

The animals of group 4 that received the commercial inactivated whole virus vaccine (MaxVac™-FLU) showed the highest titer antibody responses, as seen in the ELISA and HI results of Table 1. However, even though the animals of group 4 had roughly 1-2 fold higher HI titers relative to the animals receiving nucleic acid immunizations, this higher HI titer did not translate to a higher level of protection upon challenge (Figure 2). In fact, the animal from group 4 having the highest HI titer in the entire study was the least protected when challenged with the influenza virus.

As a result of the above-described studies, it can be seen that nucleic acid immunization to mucosal tissue via particle-mediated delivery techniques provides an immune response that is both quantitatively and qualitatively different than the responses generated by particle-mediated epidermal immunization with nucleic acids, or parenteral immunization with inactivated whole virus. Particle-mediated mucosal immunization with the pWRG1638 construct induced higher ELISA but lower HI influenza-specific antibody titers relative to particle-mediated epidermal immunization with the same construct. Further, the ability of the mucosally vaccinated animals to reduce nasal shedding of virus on days 1 and 3 of infection is consistent with a systemic mucosal immune response.

## Example 2

## Particle-Mediated Nucleic Acid Immunization Directed to Equine Mucosal Tissue

The work reported in this example was performed by a research group separate from that of the inventor here and is reported because it is supportive of the concept of the present invention.

The efficacy of mucosal nucleic acid immunization administered with a gene gun was demonstrated in ponies using the HA gene of the equine influenza strain

A/Equine/Kentucky/A/81, subcloned in a CMV promoter-based eukaryotic expression vector.

Two experimental groups, of four influenza-naive ponies each, were established. The first experimental group received a 3-dose course of particle-mediated nucleic acid immunization to epidermal tissue on days 0, 65 and 130 of the study. The second experimental group received a 3-dose course of particle-mediated nucleic acid immunization to both epidermal and mucosal tissue, also on days 0, 65 and 130 of the study. The mucosal tissue targeted was the lower side of the tongue as well as the conjunctiva and third eyelid of the animals.

Nucleic immunizations were carried out using an ACCELL® (Geniva, Madison, WI) gene gun device.

Each immunization included multiple doses of DNA delivery by gene gun. Each gene gun application delivered .5 $\mu$g of DNA. For the epidermal delivery immunizations, each immunization included 14 doses to the inguinal epidermis and 10 doses to the perineum for each animal. For the immunizations to the skin and mucosal tissue, the animals received the same skin doses plus 10 doses to the tongue and 4 doses to the conjunctiva of the third eyelid.

A challenge infection with homologous virus was administered 28 days after the final administration (on day 160 of the study) to each experimental group, and to a third group of four seronegative control ponies.

The results were that all control animals (4/4) showed clinical evidence of influenza virus infection subsequent to challenge, as did 2/4 of the animals from group 1 (receiving epidermal immunizations only). In contrast, none of the animals of group 2 that received both mucosal and epidermal nucleic immunizations (0/4) showed any clinical signs of disease. Results of influenza virus isolation on post-challenge nasal swabs demonstrated that nucleic acid immunization provided complete, or nearly complete protection from infection in two of the four horses that received epidermal and mucosal immunizations.

Example 3

Particle-Mediated Nucleic Acid Immunization in Primates to Immunodeficiency Virus

The efficacy of mucosal nucleic immunization administered with a gene gun was demonstrated in rhesus monkeys. Rhesus monkeys were given a DNA vaccine encoding a tall length gag-pol-envelope construct from simian immunodeficiency virus. The animals were vaccinated on the rectum, the tongue and on the buccal tissue. In each immunization, the gene gun was used to deliver a total of 8 $\mu$g of DNA to the tongue and cheek (buccal) tissue. Each of the animals received a prime immunization and multiple boost immunizations separated by approximately ninety days or more. In a parallel experiment, monkeys were vaccinated by administering the nucleic acid to the skin using a gene gun. Each animal received multiple boosts to the skin.

Immunized monkeys were tested to determine whether immunization induced a mucosal immune response or a systemic immune responses. The results are presented in Table 2. All four monkeys immunized mucosally showed an increase in CTL response in the mucosal gut tissue. These results indicates that the monkeys vaccinated either in the buccal or tongue tissues were able to elicit a system wide mucosal immune response as demonstrated by the existence of appropriate IgA-based CTL responses in a mucosal site, which was not a site of DNA injection. These results indicate that particle-mediated DNA delivery to mucosal tissue results in more efficient induction of mucosal-specific cellular immune responses than DNA delivery to the skin.

Mucosally-immunized monkeys also demonstrated systemic humoral and cell-mediated immune responses. Two of the three monkeys exhibited increased gag-specific peripheral or humoral blood (PBMC) CTL and three of the four monkeys showed increased IgG titers (200-400). Systemic responses were also observed in monkeys immunized by skin.

The CTL responses in LP were predominantly env-

specific. In contrast, peripheral blood CTL responses were predominantly gag-specific, regardless of route of DNA inoculation. These data suggest that mucosal immunization may differ from skin immunization in the predominant specificity of the immune responses elicited, because mucosal immunization was more efficient in eliciting env-specific immune responses.

These results indicate that in a variety of mammals the delivery of antigen encoding DNA to the mucosal tissues of the animal results in an immune response throughout the mucosal tissues of the animal even in tissues quite distance from the site of the DNA delivery. This provides a mechanism for generalized development of mucosal immune response in mammals through the use of DNA delivery to convenient tissues. Obviously, in most mammals, and in particular people, the most convenient targets for DNA delivery would be those which are least invasive, namely the tongue and the inside of the cheek.

Accordingly, novel methods for mucosal immunizations have been described. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

Table 2

| Monkey | Immunization site | Gut CTL (env) % specific lysis | PBMC CTL (gag) % specific lysis | Serum IgG (endpoint titer) |
|---|---|---|---|---|
| L978 (died) | Mucosal | 11.5[a] | dead | 400 |
| L999 | Mucosal | 14.6[a] | 28.0[d] | 200 |
| L775 | Mucosal | 12.4[b] | Negative | 0 |
| L736 | Mucosal | 10.0[b] | 18.9[d] | 400 |
| P551 | Skin | Negative*[c] | 38.4[d] | 350[e] |
| P194 | Skin | Not done | Negative | 51200[e] |
| M063 | Skin | Negative[c] | Negative | 102400[e] |
| M122 | Skin | Negative[c] | 7.6[d] | 51200[e] |
| P177 | Skin | Not done | 6.5[d] | 0[e] |
| P501 | Skin | Not done | 18.5[d] | 0[e] |
| M223 | Skin | 18.0[c] | Negative | 0[e] |

*Negative: % lysis <5.0
[a]Assayed 11/11/96, Post-boost 1 (2 immunizations)
[b]Assayed 5/19/97, Post-boost 3 (4 immunizations)
[c]Assayed 10/23/97, Post-boost 6 (7 immunizations)
[d]Assayed 8/4/98, Post-boost 8 (9 immunizations)
[e]Assayed 9/20/96, Post-boost 2, (3 immunizations)

CLAIMS

We claim:

1. A method for inducing a mucosal immune response in a mammal to a pathogen comprising the steps of

constructing copies of a nucleic acid construct capable of expressing at least one antigen from the pathogen in the cells of the mammal;

coating the nucleic acid constructs onto carrier particles;

accelerating the carrier particles into the cells of mucosal tissues of the mammal selected from the group consisting of buccal and tongue tissue, the amount of the nucleic acid and the selection of the antigen sufficient to induce a mucosal immune response in the mucosal immune system of the mammal.

2. A method as claimed in claim 1 wherein the pathogen is a virus.

3. A method as claimed in claim 1 wherein the method includes at least two repetitions at two different times of the step of delivery of the carrier particles into the mammal, one repetition being a prime immunization and the other being a boost immunization.

4. A method as claimed in claim 1 wherein the nucleic acid is DNA.

5. A method as claimed in claim 1 wherein the total amount of nucleic acid delivered into the mammal is between about 1 and 30 micrograms.

6. A method of vaccinating a mammal to impede the infection of the mammal by a virus which normally infects mammals through mucosal tissue, the method comprising the steps of

making copies of a DNA construct capable of expressing in cells of the mammal an antigen from the virus;

joining the DNA copies onto carrier particles;

physically delivering the carrier particle into the interior of cells in the mucosal tissues of the mammal, the mucosal tissues of the mammal being selected from the group consisting of tongue and buccal tissue.

7. A method as claimed in claim 6 wherein the amount of DNA delivered into the mucosal cells of the mammal is between about 1 and 30 micrograms.

8. A method as claimed in claim 6 wherein the physical delivering step is repeated at least twice at two different times, one repeat serving as a prime vaccination and the other serving as a boost.

9. A method for inducing both a mucosal immune response and a cytotoxic immune response in a mammal to a pathogen comprising the steps of:

constructing copies of a nucleic acid construct capable of expressing at least one antigen from the pathogen in the cells of the mammal;

coating the nucleic acid constructs onto carrier particles;

accelerating the carrier particles into the cells of mucosal tissue of the mammal, the mucosal tissue selected from the group consisting of buccal and tongue tissue, the amount of the nucleic acid and the selection of the antigen sufficient to induce an immune response in the mammal.

10. A method as claimed in claim 9, wherein the step of accelerating the carrier particles into the cells of the mammal is repeated one or more times.

11. A method as claimed in claim 9 wherein the pathogen is a virus.

12. A method as claimed in claim 9 wherein the method includes at least two repetitions at two different times of the step of delivery of the carrier particles into the mammal, one repetition being a prime immunization and the other being a boost immunization.

13. A method as claimed in claim 9 wherein the nucleic acid is DNA.

14. A method as claimed in claim 9, wherein the amount of nucleic acid delivered into the mammal is between about 1 and 30 micrograms.

Figure 1

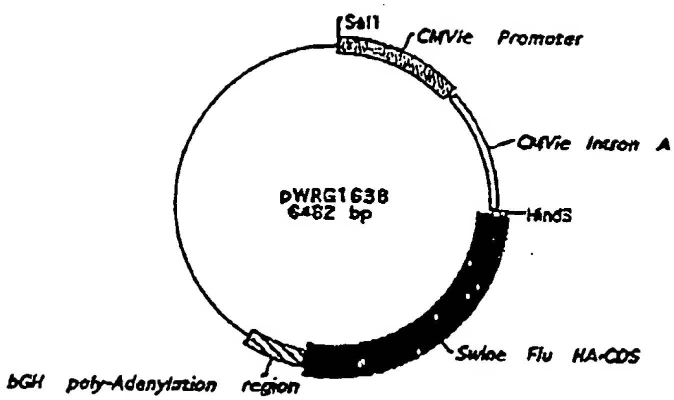
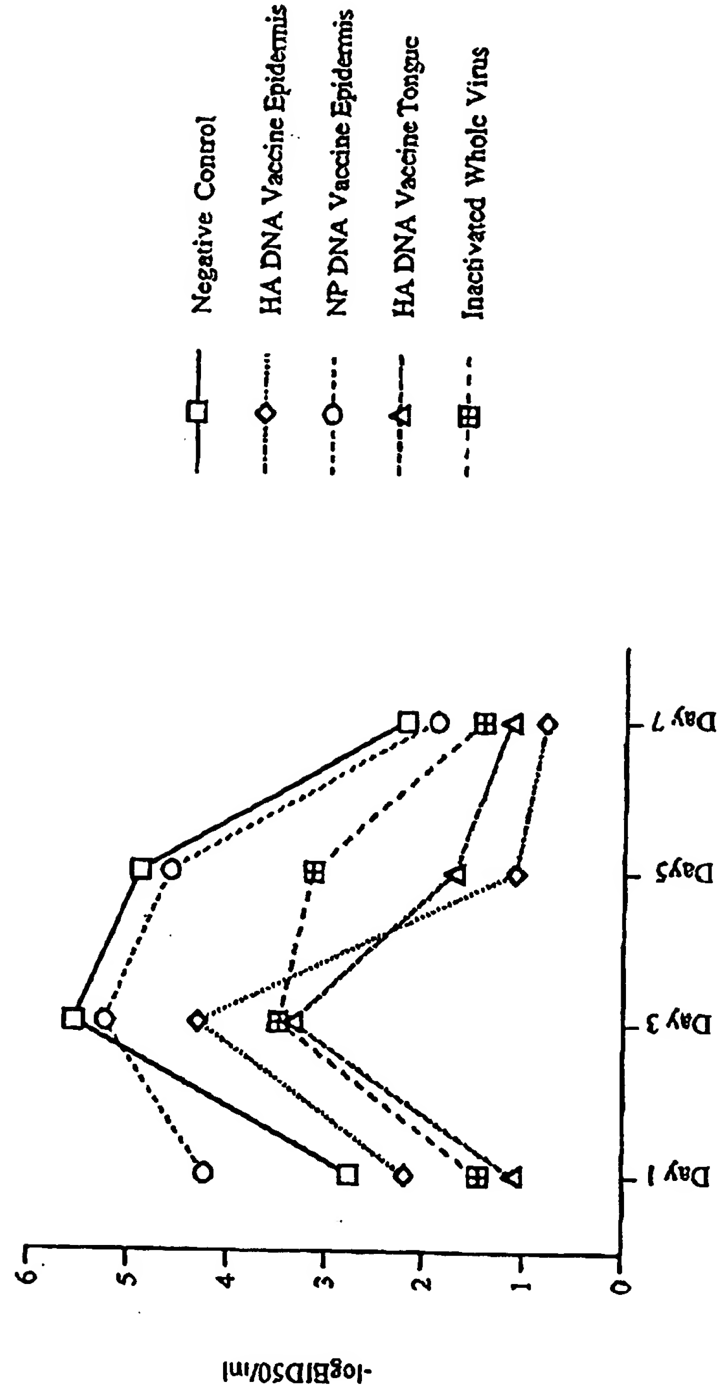

SalI
CMVie Promoter
CMVie Intron A
HindIII
pWRG1638
6482 bp
Swine Flu HA-CDS
bGH poly-Adenylation region

Figure 2


Negative Control
HA DNA Vaccine Epidermis
NP DNA Vaccine Epidermis
HA DNA Vaccine Tongue
Inactivated Whole Virus
-logBID50/ml
0
1
2
3
4
5
6
Day 1
Day 3
Day5
Day 7

| INTERNATIONAL SEARCH REPORT | International application No. PCT/US98/17637 |
|---|---|

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 31/70; C12N 15/00, 15/63; C12P 21/00
US CL :435/69.1, 172.1, 172.3; 514/44; 935/52
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.1, 172.3; 514/44; 935/52

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | STAATS et al. Mucosal immunity to infection with implications for vaccine development. Curr. Opin. Immunol. August 1994, Vol. 6, No. 4, pages 572-583, see entire document. | 1-14 |
| Y | KELLER et al. In vivo particle-mediated cytokine gene transfer into canine oral mucosa and epidermis. Cancer Gene Therapy. June 1996, Vol. 3, No. 3, pages 186-191, see entire document. | 1-14 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 12 OCTOBER 1998 | 26 OCT 1998 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>ROBERT SCHWARTZMAN for<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17637

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN:Medline, Biosis, CAPlus, Embase, WPIDS
APS
Search Terms: mucosal immunization, DNA vaccine, genetic vaccine, vector, antigen, mucosa